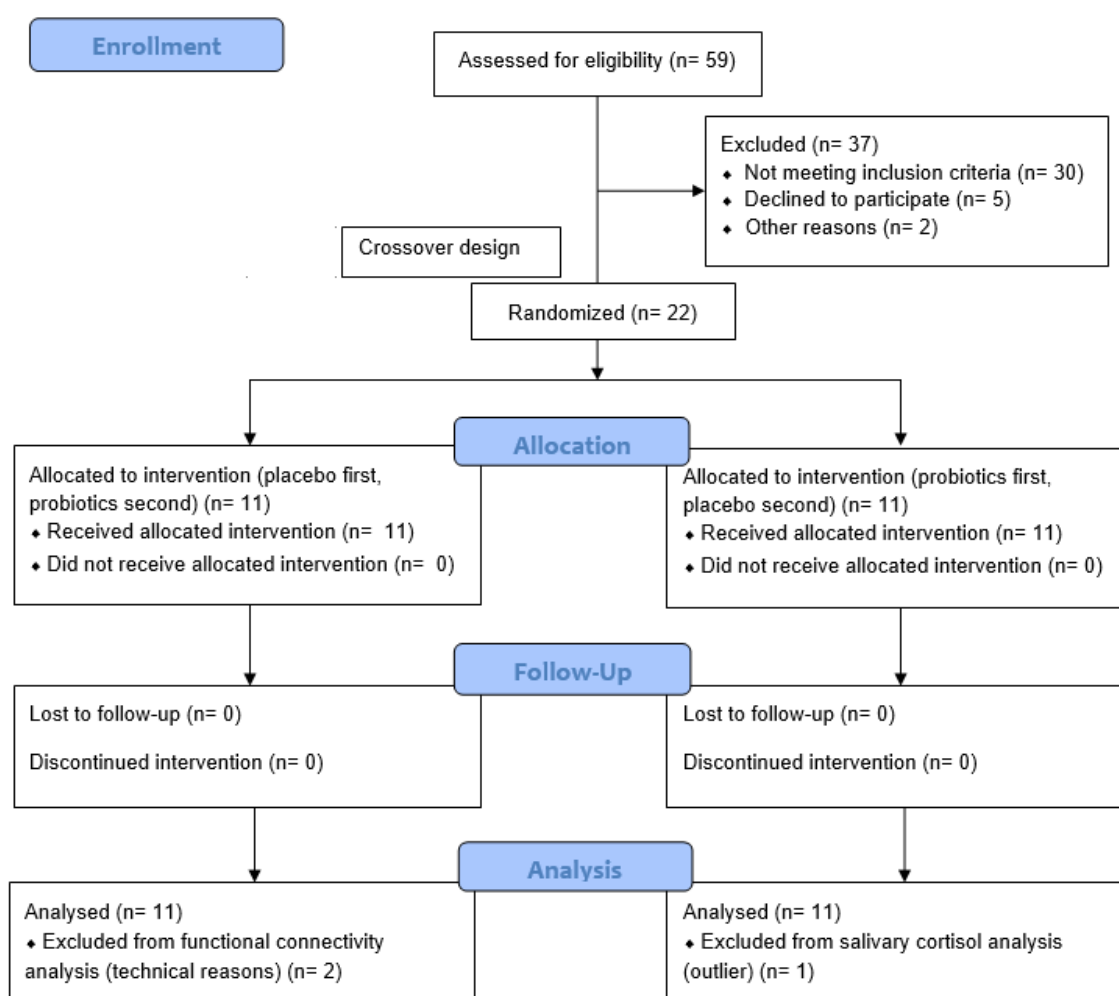


SUPPLEMENTARY INFORMATION

Probiotic mixture containing *Lactobacillus helveticus*, *Bifidobacterium longum* and *Lactiplantibacillus plantarum* affects brain responses toward an emotional task in healthy subjects: A randomized clinical trial

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* Equal contribution/ shared first authorship



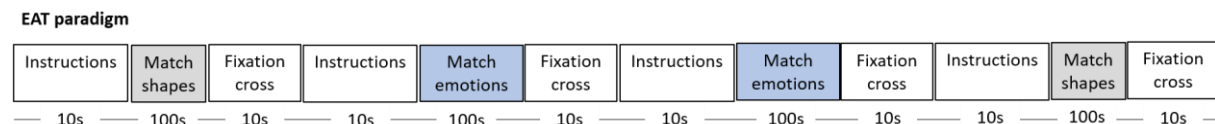
Supplementary Figure 1. Participant flow chart

Supplementary Table 1. Composition of the probiotic product

| Active Ingredients | Amount per sachet |
|--|-------------------------|
| Inulin | 500 mg |
| Magnesium | 45 mg |
| Potassium | 50 mg |
| Zinc | 9 mg |
| Glutathione | 20 mg |
| Lactoferrin | 10 mg |
| Probiotic blend: <i>L. helveticus</i> R0052 (CNCM-I-1722), <i>B. longum</i> R0175 (CNCM-I-3470), <i>L. plantarum</i> R1012 (CNCM-I-3736) | 3 x 10 ⁹ CFU |

Ingredients:

Fructose, Inulin, Magnesium Gluconate; Acidifying agent: Citric Acid; Aroma, Potassium Citrate, Mixture of live lactic cultures (*Lactobacillus Acidophilus* (Helveticus), *Bifidobacterium Longum*, *Lactiplantibacillus Plantarum*), Zinc Gluconate, Magnesium Oxide; Anti-caking agent: Silicon Dioxide; Glutathione; Sweeteners: Acesulfame K, Sucralose; Maltodextrin, Lactoferrin; Dyes: E102, E124. (underlined ingredients were also contained in the placebo)



Supplementary Figure 2. Scheme of the Emotional Attention Task (EAT)

Supplementary Information. Preprocessing of fMRI data for activity analysis

Before preprocessing, for any analyses – activity or connectivity – all DICOM images were converted to NIfTI format (using dcm2nii) using SPM12. Furthermore, the origin in structural and fMRI images were all set to the anterior commissure in order to facilitate the preprocessing and to avoid co-registration failures.

For preprocessing prior to the fMRI activity analysis, default settings were applied in the following preprocessing steps (differences from default values within parenthesis):

- Realignment of fMRI images, no wrapping was applied
- Segmentation of the structural image
- Co-registration of structural image and fMRI images. In the co-registration process the structural image was co-registered to the mean of the fMRI images
- Normalization of the fMRI images (voxel size set to 3x3x3 mm³ which is closer to the original resolution of the acquired images) to MNI space
- Normalization of the structural image (voxel size set to 1x1x3 mm³ which matched the original resolution) to the MNI space
- Smoothing (kernel = 6 mm in order to increase the specificity while losing some sensitivity, thus leading to a more conservative approach)

For each subject a simple 1st-level analysis was performed which served as a sanity check and where a typical pattern was observed using the rough contrast ME>MS. This was done to control for proper preprocessing of the data and that reasonable activations could be observed by the paradigm itself independent of any interventional effect.

A script was written which performed conversion to NIfTI file format, preprocessing and sanity check. This was to ensure that all examinations were equally preprocessed prior to the following group analysis.

Supplementary Table 2. Brain regions with relevance in literature

All brain regions that have been selected based on previous literature are presented. Those pre-defined regions that were further significantly activated in the task (ME>MS) after the placebo intervention are defined as regions of interest and are marked italic. Multiple comparison correction with Bonferroni. Significance level $p < 0.05/246$.

| Anatomical region | BNA region | ME – MS effect size | BNA region | ME – MS effect size |
|--|------------|---------------------|------------|---------------------|
| <i>Precuneus, area 31</i> | 153 | 1.790 | 154 | 2.619 |
| <i>Medial amygdala</i> | 211 | 1.653 | 212 | 1.723 |
| <i>Lateral amygdala</i> | 213 | 1.608 | 214 | 2.118 |
| <i>Rostral temporal thalamus</i> | 237 | 3.262 | 238 | 3.134 |
| <i>Rostral hippocampus</i> | 215 | 0.692 | 216 | 0.865 |
| <i>Parahippocampal gyrus, rostral area 35/36</i> | 109 | 1.015 | 110 | 0.985 |
| <i>Parahippocampal gyrus, area 28/34</i> | 115 | -0.271 | 116 | -0.050 |
| <i>Globus pallidus</i> | 221 | 0.869 | 222 | 1.600 |
| <i>Ventral caudate</i> | 219 | 2.261 | 220 | 1.901 |
| <i>Ventromedial putamen</i> | 225 | 1.727 | 226 | 2.156 |
| <i>Dorsolateral putamen</i> | 229 | 0.301 | 230 | 1.388 |
| <i>Cingulate gyrus, pregenual area 32</i> | 179 | 1.431 | 180 | 1.944 |
| <i>Cingulate gyrus, subgenual area 32</i> | 187 | -1.611 | 188 | -0.253 |
| <i>Cingulate gyrus, rostraventral area 32</i> | 177 | 1.480 | 178 | -0.126 |
| <i>Superior temporal gyrus, caudal area 22</i> | 75 | 1.704 | 76 | 2.429 |
| <i>Superior temporal gyrus, rostral area 22</i> | 79 | 1.387 | 80 | 3.097 |
| <i>Medial prefrontal cortex, median area 9</i> | 11 | 3.736 | 12 | 3.194 |
| <i>Medial prefrontal cortex, median area 10</i> | 13 | 3.774 | 14 | 5.478 |
| <i>Dorsolateral prefrontal cortex, lateral area 9</i> | 5 | 5.310 | 6 | 3.543 |
| <i>Dorsolateral prefrontal cortex, area 46</i> | 19 | 3.766 | 20 | 2.279 |
| <i>Orbitofrontal cortex, orbital area 12/47</i> | 43 | 2.593 | 44 | 4.033 |
| <i>Orbitofrontal cortex, lateral 11</i> | 45 | 0.550 | 46 | 1.789 |
| <i>Orbitofrontal cortex, medial area 11</i> | 47 | 2.016 | 48 | 2.331 |
| <i>Primary somatosensory cortex, area 2</i> | 159 | 0.305 | 160 | 1.082 |
| <i>Primary somatosensory cortex, dorsolateral area 6</i> | 7 | -0.111 | 8 | 0.255 |
| <i>Primary somatosensory cortex, medial area 6</i> | 9 | 0.035 | 10 | -0.087 |
| <i>Frontal cortex, dorsolateral area 8</i> | 3 | 0.381 | 4 | 0.848 |
| <i>Frontal cortex, ventral area 44</i> | 39 | 3.794 | 40 | 5.297 |
| <i>Insular cortex, ventral angular insula</i> | 165 | 3.105 | 166 | 3.473 |
| <i>Insular cortex, dorsal granular insula</i> | 171 | -0.408 | 172 | 1.208 |

BNA – Brainnetome atlas; ME – Match emotions; MS – Match shapes

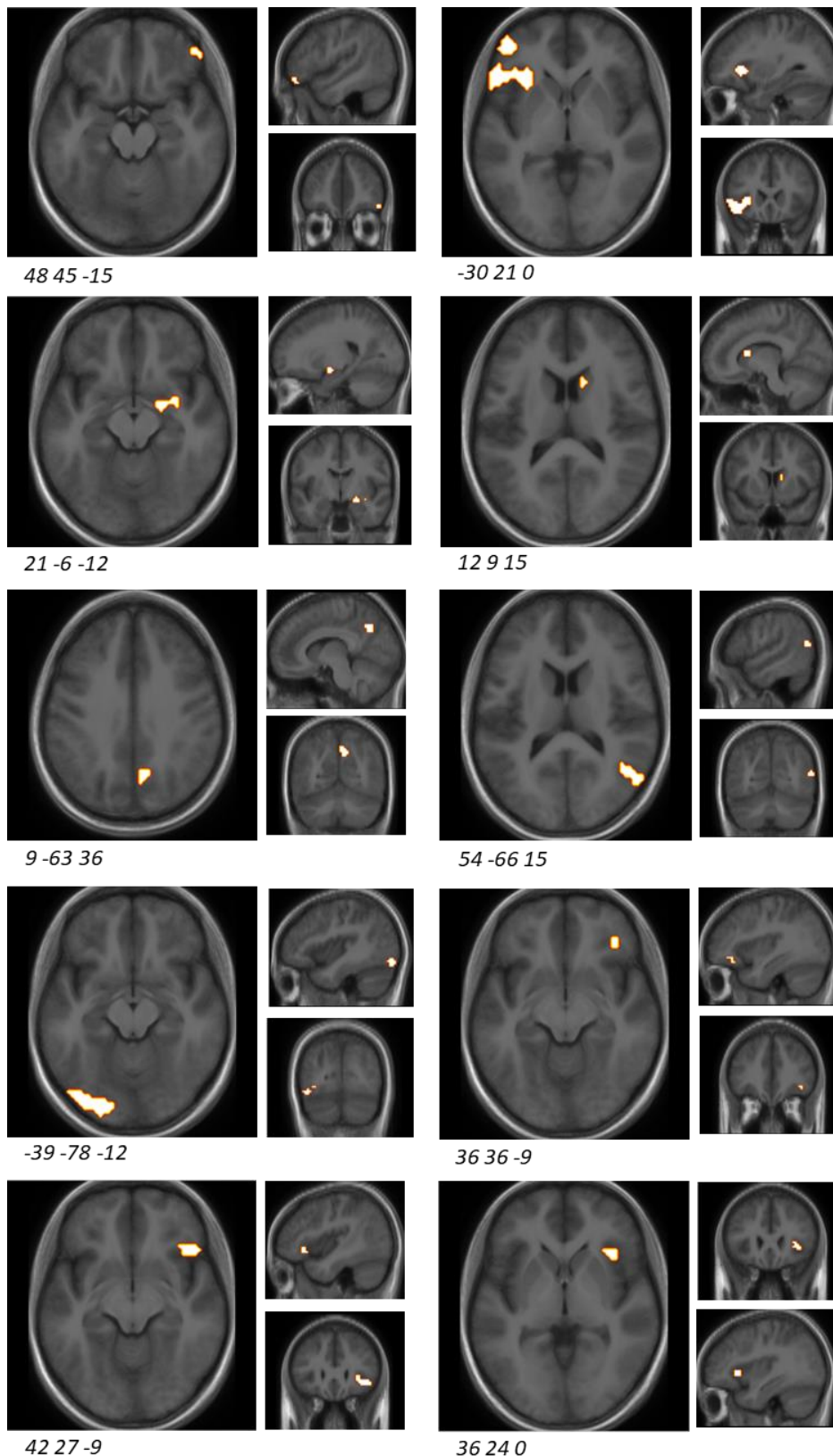
Supplementary Table 3. Clusters

Clusters that were activated by the task (ME>MS) after the placebo intervention are presented. The selected regions of interest (defined by previous literature and their involvement in the task) are marked italic. Clusters that spanned over several BNA regions were divided into sub-clusters. Multiple comparison correction with Bonferroni.

| MNI coordinates of peak (x y z) | Cluster size [mm ³] | ME – MS effect size | Anatomical region |
|--|---------------------------------|---------------------|--|
| 48 45 -15 | 378 | 6.712 | <i>Orbital gyrus, A12/47o</i> |
| -30 21 0 | 8343 | 5.880 | <i>Dorsal agranular insula</i> |
| 21 -6 -12 | 837 | 3.827 | <i>Medial amygdala</i> |
| -21 -12 -9 | 405 | 4.708 | Medial amygdala |
| 30 -27 -3 | 1269 | 3.360 | Caudal hippocampus |
| 6 66 6 | 2673 | 10.241 | Medial area 10 |
| 6 6 3 | 270 | 4.697 | Dorsal caudate |
| -9 9 6 | 675 | 4.274 | Dorsal caudate |
| -48 -51 9 | 594 | 3.899 | Caudoposterior superior temporal sulcus |
| -45 15 24 | 14472 | 6.721 | Dorsal area 44 |
| -21 69 15 | 756 | 8.414 | Lateral area 10 |
| 12 9 15 | 297 | 3.863 | <i>Dorsal caudate</i> |
| -9 63 21 | 2862 | 10.319 | Medial area 10 |
| 9 -63 36 | 1350 | 4.417 | <i>A31, area 21 (Lcl)</i> |
| 36 -54 45 | 5265 | 4.867 | Intraparietal area 7 (hIP3) |
| -33 -57 45 | 3861 | 5.367 | Rostrodorsal area 39 (hIP3) |
| -3 27 42 | 3213 | 4.910 | Medial area 9 |
| Large cluster divided into sub-clusters: | | | |
| 51 -27 0 | 459 | 3.849 | Rostral area 22 |
| 66 -42 -9 | 648 | 4.601 | Caudal area 21 |
| 57 -48 6 | 2430 | 6.036 | Dorsolateral area 37 |
| 60 -6 -15 | 1917 | 4.016 | Anterior superior temporal sulcus |
| 51 -57 -21 | 351 | 5.102 | Extreme lateroventral area 37 |
| 63 -42 -12 | 432 | 4.906 | Caudolateral of area 20 |
| 39 -60 -18 | 5427 | 11.509 | Medioventral area 37 |
| 42 -51 -21 | 4644 | 10.310 | Lateroventral area 37 |
| 51 -42 6 | 1539 | 5.125 | Rostroposterior superior temporal sulcus |
| 51 -42 9 | 945 | 6.338 | Caudoposterior superior temporal sulcus |
| 54 -66 15 | 1026 | 5.265 | <i>Caudal area 39 (PGp)</i> |
| 48 -45 12 | 1755 | 5.321 | Rostroventral area 39 (Pga) |
| 21 -93 -3 | 1539 | 7.448 | Caudal lingual gyrus |
| 24 -66 -3 | 1350 | 5.707 | Rostral lingual gyrus |
| 39 -78 0 | 2511 | 6.130 | Middle occipital gyrus |
| 42 -72 -6 | 1971 | 8.947 | Area V5/MT+ |
| 24 -93 0 | 2106 | 6.727 | Occipital polar cortex |
| 42 -78 -12 | 5427 | 12.558 | Inferior occipital gyrus |
| Large cluster divided into sub-clusters: | | | |
| -39 -75 -15 | 5157 | 12.058 | Medioventral area 37 |

| | | | |
|--|------|--------|---------------------------------|
| -39 -51 -18 | 4293 | 11.485 | Lateroventral area 37 |
| -21 -78 -6 | 1431 | 7.966 | Caudal lingual gyrus |
| -30 -93 6 | 1323 | 5.693 | Middle occipital gyrus |
| -42 -75 -6 | 675 | 8.470 | Area V5/MT+ |
| -18 -93 -6 | 1107 | 5.144 | Occipital polar cortex |
| -39 -78 -12 | 3888 | 12.113 | <i>Inferior occipital gyrus</i> |
| Large cluster divided into sub-clusters: | | | |
| 54 21 30 | 3051 | 6.866 | Inferior frontal junction |
| 45 27 21 | 648 | 6.722 | Ventral area 9/46 |
| 54 27 30 | 1215 | 8.094 | Ventrolateral area 8 |
| 54 21 27 | 2268 | 9.597 | Dorsal area 44 |
| 51 30 21 | 1107 | 8.387 | Inferior frontal sulcus |
| 57 24 21 | 1134 | 7.423 | Caudal area 45 |
| 54 42 -3 | 837 | 6.648 | Rostral area 45 |
| 51 24 -3 | 945 | 5.589 | Opercular area 44 |
| 48 15 18 | 837 | 6.545 | Ventral area 44 |
| 36 36 -9 | 432 | 4.692 | <i>Orbital area 12/47</i> |
| 42 27 -9 | 1188 | 5.906 | <i>Lateral area 12/47</i> |
| 42 9 30 | 3402 | 7.561 | Caudal ventrolateral area 6 |
| 36 24 0 | 810 | 6.117 | <i>Dorsal agranular insula</i> |

BNA – Brainnetome atlas; ME – Match emotions; MS – Match shapes; MNI – Montreal Neurological Institute



Supplementary Figure 3. Clusters selected based on their *a priori* relevance in the literature and due to their involvement in the task

Defined as regions of interest. Involvement in the task has been examined as ME – MS contrast after the placebo intervention only. Clusters are superimposed on average anatomical scans. Clusters can be identified by the coordinates of their peak (x y z).

Supplementary Table 4. Baseline characteristics

The baseline characteristics did not differ significantly between the two study arms (1. probiotics & 2. placebo vs. 1. placebo & 2. probiotics).

| | |
|---|---------------------------------------|
| Age | 24.2 ± 3.4 years |
| Male/female | 6/16 |
| BMI* | 22.3 kg/m ² (21.2 to 23.8) |
| Hospital Anxiety and Depression Scale* | |
| • Total score | 5.0 (4.0 to 8.0) |
| • Depression subscore | 1.0 (0.8 to 2.0) |
| • Anxiety subscore | 4.0 (2.0 to 6.3) |
| State and Trait Anxiety Inventory* | |
| • State subscore | 26.0 (22.0 to 30.5) |
| • Trait subscore | 28.0 (25.0 to 33.3) |
| Perceived Stress Scale (total score)* | 9.0 (5.8 to 12.3) |
| Quality of life (based on EQ-5D-5L index value)* | 80.5% (75.0 to 90.0) |

*For BMI and all questionnaire scores median and interquartile range (IQR 25 to 75) are reported

BMI – Body mass index

Supplementary Table 5. Baseline diet

Subjects were asked to fill in a 3-day food diary. The three days have been analyzed separately, but average nutrients of the three days are reported. Median and IQR (25 to 75) are reported. The background diet of all subjects of the two study arms (1. probiotics & 2. placebo vs. 1. placebo & 2. probiotics) did not differ significantly.

| Protein [g] | Fat [g] | Carbohydrates [g] | Alcohol [g] | Fibers [g] | Water [g] |
|------------------------|--------------------------|--------------------------|--------------------|------------------------|---------------------------|
| 84.0 (72.0 to 90.1) | 107.7 (90.3 to 117.5) | 210.2 (182.5 to 235.6) | 0.0 (0.0 to 0.0) | 23.5 (18.6 to 27.8) | 1848.0 (1554.0 to 2055.0) |

Supplementary Table 6. Physical activity

Physical activity was recorded during one week before the start and during the last week of each intervention period by Actigraphy (Actiwatch spectrum-pro and Actiware 6.0 software, Philips Respironics, Andover, MA, USA) and averaged per week. Physical activity did not differ significantly between the two study arms nor changed significantly during the course of the study. Median and interquartile range (IQR 25 to 75) are reported.

| One week at 1. baseline [counts/minute] | Last week of 1. intervention [counts/minute] | One week at 2. baseline [counts/minute] | Last week of 2. intervention [counts/minute] |
|--|---|--|---|
| 188.2 (146.2 to 218.9) | 185.0 (143.2 to 211.9) | 182.4 (146.5 to 214.2) | 183.0 (153.8 to 261.2) |

Supplementary Table 7. Adverse events

The total number of adverse events is reported, the number of those events which were suspected to be related to the intervention or possibly related to the intervention are shown in brackets: total number (suspected/possible).

| Symptom | Placebo | 4 weeks after placebo | Probiotic | 4 weeks after probiotic |
|---------------------|----------|-----------------------|-----------|-------------------------|
| Abdominal pain | 2 (0/2) | 0 (0/0) | 1 (0/1) | 0 (0/0) |
| Diarrhea | 0 (0/0) | 1 (0/0) | 2 (0/2) | 0 (0/0) |
| Bloating | 1 (0/1) | 0 (0/0) | 1 (0/1) | 0 (0/0) |
| Nausea | 1 (0/1) | 0 (0/0) | 2 (0/2) | 1 (0/0) |
| Headache | 12 (4/1) | 1 (0/0) | 5 (0/2) | 0 (0/0) |
| Cold | 14 (0/0) | 0 (0/0) | 6 (0/1) | 3 (0/0) |
| Sore throat | 1 (0/0) | 0 (0/0) | 0 (0/0) | 1 (0/0) |
| Fever | 1 (0/0) | 0 (0/0) | 1 (0/0) | 0 (0/0) |
| Sleep problems | 2 (0/2) | 0 (0/0) | 1 (0/1) | 0 (0/0) |
| Depressive symptoms | 0 (0/0) | 1 (0/1) | 0 (0/0) | 1 (0/1) |
| Anxiety symptoms | 0 (0/0) | 0 (0/0) | 1 (0/1) | 0 (0/0) |
| Pain in the knee | 0 (0/0) | 0 (0/0) | 2 (0/0) | 0 (0/0) |
| Fungal infection | 1 (0/1) | 0 (0/0) | 0 (0/0) | 0 (0/0) |
| Pollen allergy | 1 (0/0) | 0 (0/0) | 4 (0/0) | 0 (0/0) |

Supplementary Table 8. Compliance by intervention period

All subjects consumed 90% or more of the study product.

| | Daily consumption of the study product | One single day consumption missed during second intervention | One day consumption missed in both interventions | Several days consumption missed during first intervention and one day missed during second intervention |
|--------------------|--|--|--|---|
| Number of subjects | 14 (63.6%) | 4 (18.2%) | 2 (9.1%) | 2 (9.1%)* |

*Two subjects forgot to consume the intervention product on 3 and 4 days, respectively, during the first intervention period, with a maximum of two missing consecutive days.

Supplementary Table 9. Compliance by product

All subjects consumed 90% or more of the study product.

| | Daily consumption of the study product | One single day consumption missed | Several days consumption missed |
|------------|--|-----------------------------------|---------------------------------|
| Placebo | 14 (63.6%) | 8 (36.4%) | 0 (0.0%) |
| Probiotics | 18 (81.8%) | 2 (9.1%) | 2 (9.1%)* |

*Two subjects forgot to consume the intervention product on 3 and 4 days, respectively, with a maximum of two missing consecutive days.

Supplementary Information. Whole-brain analysis based on BNA regions

Additionally to a cluster-based analysis focusing on functionally altered areas a whole-brain analysis was performed based on anatomically defined brain regions. The intensity of signals of all voxels of individual brainnetome atlas (BNA) regions were averaged per brain volume, keeping the time course of the signal. The regions of significant p-values were defined using the BNA atlas. Among those regions being involved in the task, n=26 overlapped with the literature-based selection and were selected for the region-based analysis (Supplementary Table 2) investigating the intervention effect.

fMRI activity profiles for BNA regions were computed as averages of the composing voxels prior to modeling them according to equation 1 and 2 in the same way as done for the cluster-based analysis.

Comparing the probiotic and the placebo intervention, BNA region-based analysis showed significant ($p < 0.1$) changes in activation (ME versus MS) in several regions that were among the predefined regions of interest (without correction for multiplicity) (Supplementary Table 10). Reduced activation (probiotics < placebo) was observed in the parahippocampal gyrus, hippocampus, amygdala, basal ganglia and cingulate gyrus. Increased activation (probiotics > placebo) was seen in the orbital gyrus and precuneus.

Supplementary Table 10. BNA regions that were found to be associated with significant ($p < 0.1$) changes in brain activity between both interventions before multiplicity correction, during the EAT paradigm. Eleven of these regions were predefined regions of interest (*italic*).

| BNA region | ME – MS in probiotic – placebo p-value* | ME – MS in probiotic – placebo effect size | Anatomical region |
|------------|---|--|--|
| 115 | 0.006 | -1.140 | <i>Parahippocampal gyrus, area 28/34</i> |
| 30 | 0.007 | 2.014 | Inferior frontal gyrus, dorsal area 44 |
| 29 | 0.012 | 2.080 | Inferior frontal gyrus, dorsal area 44 |
| 52 | 0.018 | 2.124 | <i>Orbital gyrus, lateral area 12/47</i> |
| 220 | 0.026 | -2.150 | <i>Basal ganglia, ventral caudate</i> |
| 213 | 0.030 | -2.098 | <i>Amygdala, lateral amygdala</i> |
| 221 | 0.037 | -0.948 | <i>Basal ganglia, globus pallidus</i> |
| 149 | 0.046 | 0.914 | <i>Precuneus, medial area 5</i> |
| 81 | 0.046 | 1.075 | Middle temporal gyrus, caudal area 21 |
| 223 | 0.050 | -1.421 | Basal ganglia, nucleus accumbens |
| 199 | 0.057 | -1.190 | Lateral occipital cortex, middle occipital gyrus |
| 117 | 0.069 | -1.240 | <i>Parahippocampal gyrus, area TI</i> |
| 215 | 0.075 | -1.390 | <i>Hippocampus, rostral</i> |
| 64 | 0.075 | 1.429 | Precentral gyrus, caudal ventrolateral area 6 |
| 87 | 0.081 | 0.597 | Middle temporal gyrus, anterior superior sulcus |
| 219 | 0.085 | -1.274 | <i>Basal ganglia, ventral caudate</i> |
| 211 | 0.088 | -1.320 | <i>Amygdala, medial</i> |
| 178 | 0.096 | -1.343 | <i>Cingulate gyrus, rostroventral area 24</i> |

*After correction for multiple testing using Bonferroni, the changes in activation in all BNA regions ($p > 0.05/246$) and the predefined ROIs ($p > 0.05/26$) did not remain statistically significant.

BNA – Brainnetome atlas, EAT – Emotional Attention Task, ME – Match emotions, MS – Match shapes